2003. The Journal of Arachnology 31:412–420

THE FIRST DNA PHYLOGENY OF FOUR SPECIES OF *MESOBUTHUS* (SCORPIONES, BUTHIDAE) FROM EURASIA

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ABSTRACT. The first molecular phylogeny is presented for four species of the scorpion genus *Mesobuthus*, based on DNA sequences of three gene fragments (two mitochondrial and one nuclear protein coding gene, ~1 kb). The inferred phylogeny based on a pooled maximum likelihood analysis revealed a clear deep splitting between the "western clade" consisting of *M. gibbosus* and *M. cyprius* (Greece/Anatolia, Cyprus) and the "eastern clade" consisting of *M. eupeus* and *M. caucasicus* (Anatolia/Central Asia). The species *M. caucasicus* (recently placed in the genus *Olivierus* Farzanpay 1987) groups monophyletically within *Mesobuthus*; thus, the genus *Olivierus* is synonymized here with *Mesobuthus*. Sequences of *M. eupeus* and *M. caucasicus* sampled mainly from Kazakhstan and Uzbekistan are highly structured, indicating the possible existence of multiple species.

Keywords: Scorpions, Buthidae, *Mesobuthus*, phylogeny, DNA, 16S, *cox*1, protein kinase, biogeography

The genus *Mesobuthus* Vachon 1950 (Scorpiones, Buthidae) currently includes 12 species (Fet & Lowe 2000; Gantenbein et al. 2000b); its type species is *Mesobuthus eupeus* (C.L. Koch 1839). Except for *M. gibbosus* (Brullé 1832), which is found in the Balkans and Turkey, the diversity of this genus is concentrated in Asia. Numerous species and subspecies are distributed from Turkey to Korea, with the centers of diversity in Central Asia and Iran. *Mesobuthus* species are the most common and abundant scorpions in a variety of arid habitats, from sand deserts to high mountains over 3000 m (Fet 1989, 1994). They are found up to 50°N in Kazakhstan, the northern limit of the natural range of the Old World scorpions (Gromov 2001).

Although Buthidae are the most diverse and medically important family of scorpions (Fet & Lowe 2000), there has been no attempt so far to produce a phylogenetic analysis of this family. Especially powerful are phylogenies based on DNA sequence data in combination with morphology (Gantenbein et al. 1999a, 2000a; Fet et al. 2001). Our first applications of this technique in Buthidae refer to the genera *Buthus* Leach 1815 (Gantenbein et al. 1999b; Gantenbein & Largiadèr 2003) and *Centruroides* Marx 1890 (Gantenbein et al. 2001; Towler et al. 2001); we also published a pilot phylogeny of 17 buthid genera (Fet et al. 2003). Molecular markers helped to define island species, where neither the biological species concept nor any other species concept can be applied (Gantenbein et al. 2000b, 2001).

METHODS

The currently studied available material belonged to four species: *Mesobuthus gibbosus* (Greece, Turkey), *M. cyprius* Gantenbein & Kropf 2000 (Cyprus), *M. eupeus* (C.L. Koch 1839) (Turkey, Kazakhstan, Turkmenistan, Uzbekistan, China), and *M. caucasicus* (Nordmann 1840) (Kazakhstan, Turkmenistan, Uzbekistan) (see Table 1 for locality information). For DNA analyses, the total DNA was extracted from fresh or preserved (94–98% ethanol) muscle tissue using a standard phenol/chloroform and precipitation method (Sambrook et al. 1989). We amplified a ca. 450 base pair (bp) fragment of the 16S rRNA mitochondrial (mt) DNA by polymerase chain reaction (PCR) using the primers and condi-

Table 1.—Sampling sites and country of origin of *Mesobuthus* species used in this study.

Abbreviation	Country	Locality	Coll.	EMBL accession nos.		
				16S	*cox*I	PK
M. caucasicus (Nordmann 1840)						
*Mca*KZa1	Kazakhstan	Kapchagai	Ch. Tarabaev, 1990	AJ550674	AJ550692	AJ550713
*Mca*KZb1	Kazakhstan	Baigakum	V. Fet & A. Gromov, 25-V-2002	AJ550675	AJ550693	AJ550714
*Mca*UZa1	Uzbekistan	Bukhara	V. Fet & A. Gromov, 20-IV-2002	AJ550676	AJ550694	AJ550715
*Mca*UZb1	Uzbekistan	Jarkurgan	V. Fet & A. Gromov, 26-IV-2002	AJ550677	AJ550695	AJ550716
*Mca*UZc1	Uzbekistan	Karakalpak Steppe, Ferghana	V. Fet & A. Gromov, 20-V-2002	AJ550678	AJ550696	AJ550717
*Mca*UZd1	Uzbekistan	Babatag Mountains	V. Fet & A.Gromov, 30-IV-2002	AJ550679	AJ550697	AJ550718
M. cyprius Gantenbein & Kropf 2000						
*Mcy*CYa1	Cyprus	Tepebasi	A. Scholl, 27-IX-1997	AJ550680	AJ550698	AJ550719
*Mcy*CYb1	Cyprus	Kantara	A. Scholl, 20-V-1998	AJ550681	AJ550699	AJ550720
M. eupeus mongolicus (Birula 1911)						
MeuGobi1	China	Gobi Desert	A. Davidson, 30-VII-1998	AJ550682	AJ550700	AJ550721
M. eupeus eupeus (C.L. Koch 1839)						
*Meu*TRa1	Turkey	Gülsehir, Central Anatolia	A. Scholl, 28-V-1998	AJ550688	AJ550701	AJ550722
*Meu*TRb1-2	Turkey	Cemilköy, Central Anatolia	A. Scholl, 31-V-1998	AJ550689–90	AJ550702–03	AJ550723–24
M. eupeus thersites (C.L. Koch 1839)						
*Meu*KZa1	Kazakhstan	Bakanas	A. Gromov, 2-5-VI-2000	AY228141	AJ550704	AJ550725
*Meu*KZb1	Kazakhstan	Baigakum	V. Fet & A. Gromov, 23-V-2002	AJ550684	AJ550705	AJ550726
*Meu*KZc2	Kazakhstan	Karatau Mountains	V. Fet & A. Gromov, 27-V-2002	AJ550685	AJ550706	AJ550727
*Meu*TU1a	Turkmenistan	Repetek, Karakum	V. Fet & A. Gromov, 15-18-IV-2002	AJ550686	AJ550707	AJ550728
*Meu*UZa1	Uzbekistan	Zarafshan, Kizylkum	A. Gromov, 18-IV-1998	AJ550687	AJ550708	AJ550729

Table 1.—Continued.

Abbreviation	Country	Locality	Coll.	EMBL accession nos. 16S	*cox*I	PK
*Meu*UZb1	Uzbekistan	Babatag Mountains	V. Fet & A. Gromov, 29-IV-2002	AJ550683	AJ550709	AJ550730
Mesobuthus gibbosus (Brullé 1832)						
*Mgi*GRa1	Greece	Mathia, Peloponnesos	I. & B. Gantenbein, 18-III-1998	AJ402571	AJ550710	AJ550731
*Mgi*GRb1	Greece	Igoumenitsa, Epirus	I. & B. Gantenbein, 28-IV-1998	AJ550691	AJ550711	AJ550732
*Mgi*TRa1	Turkey	Avanos, Central Anatolia	A. Scholl, 28-V-1998	AJ402587	AJ550712	AJ550733
Outgroup: *Androctonus australis* (Linnaeus 1758)						
AanTNa1	Tunisia	Nefta	A. Scholl, 27-IV-1999	AJ506868	AJ506919	AJ550734

tions of Gantenbein et al. (1999). For the partial amplification of the cytochrome oxidase I (*cox*I) gene we used the primers *LCO* (Folmer et al. 1994) and *Nancy* (Simon et al. 1994) which amplify a ~850 bp fragment (positions ~30–850 of the *Drosophila yakuba* sequence; Flybase: FBgn0013179). We used the following PCR profile: initial denaturation 94 °C for 4 min followed by 40 cycles of 25 s at 94 °C, 20 s at 51 °C, and 90 s at 72 °C. In addition, we designed new buthid-specific PCR primers from a clone (03B09) of an EST library of *Mesobuthus gibbosus* (unpublished data, Gantenbein et al., in preparation) to amplify a ~360 bp fragment of the nuclear protein kinase (PK) gene (Flybase locus CG11221, identified from BLASTX against the *Drosophila* protein database, similarity 43%, Expectation = 5e–14). The primers were *03B09for* 5′-TCT GAT GTA TGG CAG ATG GCA ATG-3′ and *03B09rev* 5′-CGA ACT CAA GAT CCA CTC CTG TAC TCG-3′. We used the same PCR profile as for *cox*I. PCR primers were removed by polyethylene glycol (PEG 8000) precipitation, and templates were directly sequenced on one strand using one of the PCR primers and DYEnamic ET Dye Terminator Kit (Amersham Biosciences). For *cox*I and PK we used the forward PCR-primers for sequencing, and for the 16S we used the same primer as in Gantenbein et al. (1999). Sequencing reactions were ethanol/sodium-acetate precipitated and run on an ABI377XL sequencer (Applied Biosystems, Foster City, CA). All sequences were checked manually for sequencing errors. All sequences were deposited in the EMBL nucleotide sequence database (http://www.ebi.ac.uk). As outgroup, we used the Old World buthid *Androctonus australis* (L. 1758) from Nefta, Tunisia (AauTNa1), which is a suitable outgroup for the genus *Mesobuthus* as indicated from

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Figure 1.—Maximum Likelihood (ML) tree of *Mesobuthus* species from southern Europe, Western and Central Asia inferred from three combined DNA sequence fragments of the mitochondrial 16S, *cox*I and the nuclear PK regions, 1,095 bp (-ln Likelihood was 5505.86). The DNA substitution model was TRN + Γ + I (Tamura & Nei 1993); base frequencies: $\pi_A = 0.29$, $\pi_T = 0.37$, $\pi_C = 0.14$, R_{matrix} = (A-G = 6.70, A-C = A-T = G-T = 1, C-T = 3.56), gamma shape parameter $\alpha = 0.53$, and proportion of invariable sites = 0.37, respectively. The tree was rooted using the outgroup species *Androctonus australis* (AauTNa1). Numbers at nodes refer to bootstrap support given as percentage from 1,000 pseudo replicates by neighbour-joining of ML distances.

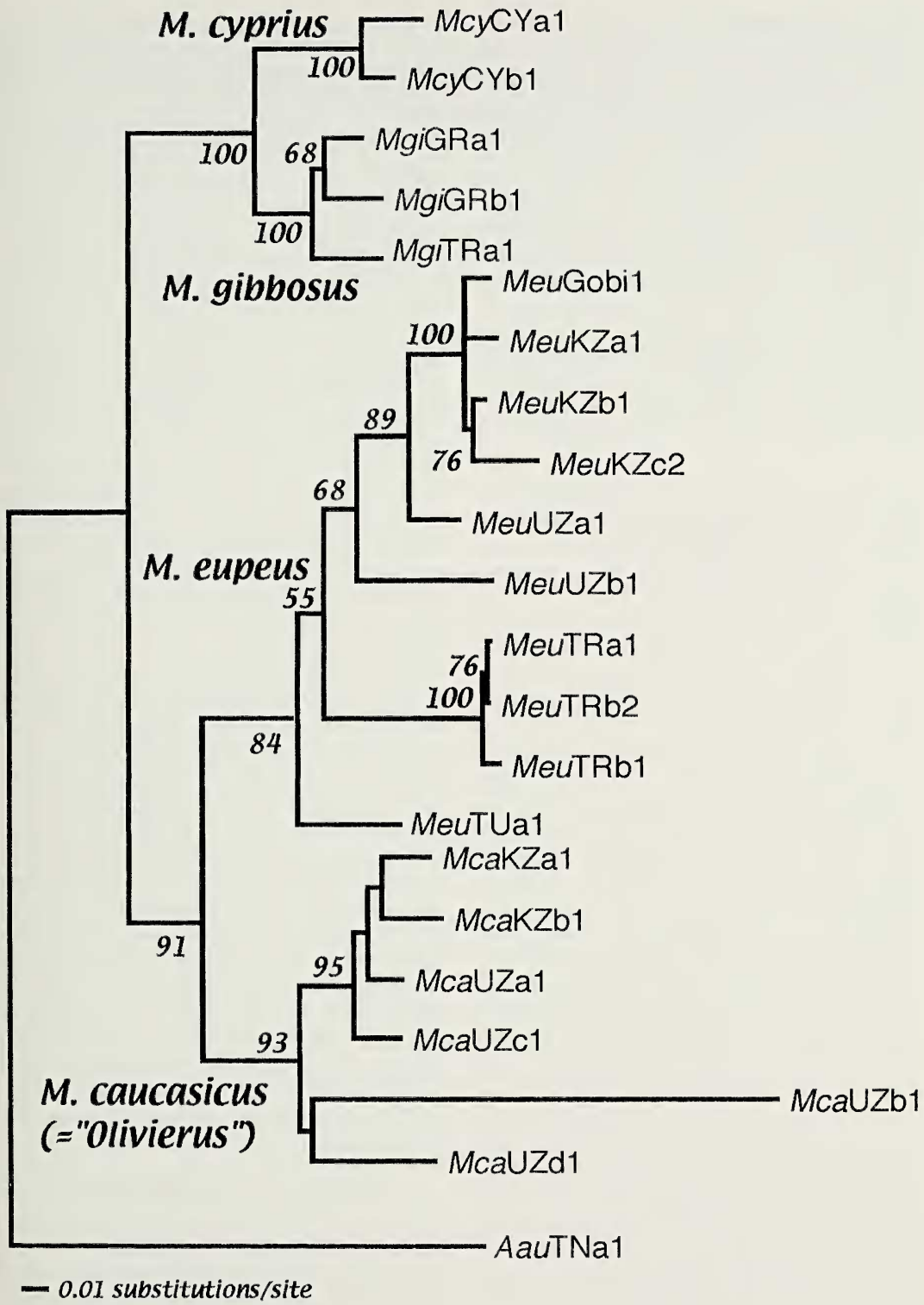
M. cyprius
McyCYa1
100
McyCYb1
100
68
MgiGRa1
MgiGRb1
100
MgiTRa1
M. gibbosus
MeuGobi1
100
MeuKZa1
89
MeuKZb1
76
MeuKZc2
68
MeuUZa1
M. eupeus
MeuUZb1
55
MeuTRa1
76
100
MeuTRb2
84
MeuTRb1
MeuTUa1
McaKZa1
McaKZb1
91
95
McaUZa1
93
McaUZc1
M. caucasicus
(="Olivierus")
McaUZb1
McaUZd1
AauTNa1
0.01 substitutions/site

Table 2.—Estimates of average rates of synonymous substitutions (K_S) (lower left) and non-synonymous (K_A) (upper right) substitution per site among four species of *Mesobuthus* of two protein-coding gene fragments. Diagonals show average K_S and K_A (in parentheses) among haplotypes within species. Estimates of nucleotide diversity π for each species (Nei & Li 1979) are given in the last column.

	cyprius	*caucasicus*	*eupeus*	*gibbosus*	diversity π
coxI locus					
cyprius	0.11 (0.009)	0.297	0.024	0.006	0.037
caucasicus	0.844	0.482 (0.030)	0.034	0.023	0.098
eupeus	0.887	0.753	0.377 (0.026)	0.023	0.089
gibbosus	0.538	0.892	0.692	0.223 (0.004)	0.048
PK locus					
cyprius	0.038 (0.018)	0.106	0.013	0.013	0.032
caucasicus	0.093	0.022 (0.000)	0.004	0.004	0.008
eupeus	0.073	0.032	0.008 (0.000)	0.009	0.002
gibbosus	0.04	0.082	0.064	0.037 (0.009)	0.017

morphology (Vachon 1952). Voucher specimens will be deposited in the Natural History Museum, Bern, Switzerland (NHMBE).

The resulting 22 16S DNA sequences were aligned using ClustalX (Thompson et al. 1997) and by eye. We applied Maximum Likelihood (ML) to the DNA sequence data. The alignment was 344 bp long. The alignment was deposited in the EMBL nucleotide database (ALIGN_000522). Ambiguities and gaps were stripped out (Swofford et al. 1996), leaving 302 bp. The alignment of the *cox*I and the PK fragments was unambiguous because of the open reading frames (ORF). In order to choose the most appropriate DNA model of nucleotide substitution, we calculated hierarchic likelihood ratio test statistics using MODELTEST 3.06 (Posada & Crandall 1998; Huelsenbeck & Rannala 1997) which is implemented in PAUP* 4.0b10 (Swofford 1998) and calculates the hierarchical likelihood ratio statistics (LRT) of 56 different substitution models based on a NJ tree using JC69 distances (Jukes & Cantor 1969). The rate heterogeneity among sites was assumed to follow a gamma distribution (shape parameter α was ML-estimated) with four categories, each represented by its mean (Yang 1996). The ML-estimated parameters and the model are given in the legend of Fig. 1.

The tree topology found with ML of the pooled mitochondrial data (16S & *cox*I) was not significantly different from the topology inferred from the nuclear PK gene if assessed by the two-tailed K-H-test ($-\ln L_{(\text{treePK}|\text{PKdata})} = 618.49$; $-\ln L_{(\text{tree16S\&}cox\text{I}|\text{Pkdata})} = 632.50$; $P = 0.16$) with re-estimation of maximum likelihood by non-parametric bootstrapping (RELL) (Kishino & Hasegawa 1989; Kishino et al. 1990). Thus, we pooled the nuclear and mitochondrial data, leaving 1,095 bp (475 bp of the *cox*I and 318 bp of the PK, excluding gaps and ambiguities).

In a further step, the molecular clock hypothesis (i.e., equal rates across all sequences) was tested using the χ^2 approximated likelihood ratio test statistics with OTU's−2 degrees of freedom (df = 22–2 = 20) which was rejected with a P-value < 0.01 (Huelsenbeck & Crandall 1997). We explored the tree space by 100 heuristic tree searches using the Tree-Bisection-Reconnection (TBR) algorithm and by randomizing the order of the sequence input in PAUP*. Phylogenetic trees were rooted using *Androctonus australis* as an outgroup. Statistical confidence of phylogenies was assessed using the bootstrap procedure (1,000 pseudoreplicates) (Felsenstein 1985) using PAUP*. These distances were usually ~15 % between *Mesobuthus* species and go up to ~20 % if *Mesobuthus* sequences were compared to the outgroup.

RESULTS AND DISCUSSION

The three analyzed DNA fragments contained considerable polymorphism among but also within species as estimated using Nei & Li's (1979) nucleotide diversity π and the amount of synonymous versus non-synonymous substitutions of coding regions (Jukes & Cantor 1967) (Table 2). We found 153 mutations out of 475 bp (of which 29 were replace-

ment changes) in the *cox*I fragment, and 19 mutations out of 318 bp (of which 8 were replacement changes) in the PK gene (excluding the outgroup). The 16S fragment contained 131 polymorphic sites out of 302 bp.

The recovered phylogeny (Fig. 1) showed high support for all four included species. This phylogeny allows to address several important taxonomic and evolutionary issues pertaining to the genus *Mesobuthus*. First of all, the phylogeny demonstrates a deep split between the "western clade" of *M. gibbosus* and *M. cyprius* and the "eastern clade" of *M. eupeus* and *M. caucasicus* (Fig. 1); each clade is well supported (100% and 92% bootstrap, respectively). Within the "eastern clade," there is a strong support for currently accepted species *M. eupeus* (87%) and *M. caucasicus* (94%). The *Mesobuthus caucasicus* sequences are nested within the genus *Mesobuthus* as a sister group to *M. eupeus*. This observation is important as the generic placement of *M. caucasicus* has been controversial since Farzanpay (1987) created a separate monotypic genus *Olivierus* for this species. As our molecular data show, this new genus is paraphyletic with respect to *Mesobuthus* (Fig. 1). Fet & Lowe (2000) listed *Olivierus* as a valid genus but noticed that it was created without any solid justification or revision. The only characters (number of granules on movable finger of pedipalp chela) that Farzanpay (1987) briefly quoted (in Farsi) in support of *Olivierus*, were borrowed from Vachon (1958) species-level descriptions, and are not diagnostic for a genus. Further, our molecular data do not support monophyly of *Olivierus*. Therefore, we propose to list this genus as a synonym of *Mesobuthus*, as was traditionally accepted by all authors before Farzanpay (1987): *Olivierus* Farzanpay 1987 = *Mesobuthus* Vachon 1950, NEW SYNONYMY.

Another observation refers to the subspecific structure of *M. eupeus*, the type species of the genus *Mesobuthus*. This species is extremely polymorphic, and has 14 formally valid subspecies ranging from Turkey to China (Vachon 1958; Fet 1989; Fet & Lowe 2000), with most subspecies described from Iran. The nominotypic subspecies *M. e. eupeus* (C.L. Koch 1839) is found in the Caucasus and Turkey, while most populations from Central Asia are classified as *M. e. thersites* (C.L. Koch, 1839). In our analysis, the nominotypic subspecies (Turkey) is highly supported (bootstrap 100%) while the Central Asian sequences present a more complicated case. A separate clade exists for a sand desert population from Turkmenistan (MeuTUa1), as opposed to the clade of several populations from Uzbekistan, Kazakhstan and China (bootstrap 68%). This can be an indication of an ancient separation between southern and northern desert forms, possibly valid at least at the subspecies level (Fet 1994). The further phylogenetic analysis could result in elevating these subspecies to the species level. It is interesting that already Birula (1917) grouped all subspecies of *M. eupeus* into two species groups ("sections"), "*eupeus*" and "*thersites*"; however, status of these groups was never examined.

Within *M. caucasicus*, a very strongly supported clade (bootstrap 98%) groups populations from central Uzbekistan (Bukhara, Ferghana) and Kazakhstan, while those from southern Uzbekistan (Jarkurgan, Babatag) group outside. It remains to be seen if genetic separation in this case is matched by the morphological variation, as there are several subspecies described from Central Asia as well.

In addition, in our phylogeny the northern Central Asian populations of both *Mesobuthus eupeus* and *M. caucasicus* (Kazakhstan) appear to be derived compared to the southern populations of both species (Uzbekistan); this could be the result of progressive Tertiary aridization and spreading of the arid scorpion species from south to north to the sand and clay deserts (Fet 1994).

The presented data also allow a calibration of a molecular clock using the separation of Cyprus from the Anatolian mainland (5.2 Mya) after the Messinian salinity crisis, during which gene flow between island and mainland populations could have been possible. The Mediterranean Basin was refilled within only 100 yrs, which provides an excellent calibration point for a molecular clock (Hsü et al. 1977; Gantenbein & Largiadèr 2002). Thus, the sequence divergence between Anatolia and Cyprus was estimated to 0.09 ± 0.01 (0.10 ± 0.01 for 16S), which results in a sequence divergence rate of 0.017 per My. This rate estimate is somewhat higher than previous estimates in scorpions for *Mesobuthus gibbosus* (Gantenbein & Largiadèr 2002) but lies absolutely in the range of scorpions such

as *Buthus occitanus* and *Centruroides* (Buthidae) and other invertebrates such as butterflies, beetles and crickets (Brower 1994; Fleischer et al. 1998; Gómez-Zurita et al. 2000; Gantenbein et al. 2001; Gantenbein & Largiadèr 2003).

The genus *Mesobuthus* was created when Vachon (1950) initiated a large-scale "splitting" revision of the traditional genus *Buthus* Leach 1815. Its composition is still controversial. For instance, several Indian species are classified currently in this *Mesobuthus* but their generic identity is unclear (Fet & Lowe 2000). Separate species have been only recently described from Cyprus (Gantenbein et al. 2000b) and confirmed for northern Israel (Fet et al. 2000). Numerous subspecies exist in *M. eupeus* and *M. caucasicus* but morphological characters are inconclusive as for their relationship and taxonomic status. Further application of new molecular markers will facilitate our understanding of taxonomy and evolution of this common scorpion genus.

ACKNOWLEDGMENTS

We are thankful to our late colleagues, Gary A. Polis (California, USA) and Chingiz Tarabaev (Almaty, Kazakhstan) for their help and support in the scorpion work. We dedicate this contribution to their lives, which inspired many young biologists all over the world. We are grateful to A. Scholl and A. Davidson for donation of the specimens used in this study, and to Mark D. Barker and W. Ian Towler for their skilled help in the lab. B.G. was supported by a SNF grant (SNF-83EU–065528) and the Royal Society (R-36579) and V.F. by a National Geographic Society Research and Exploration Fund (grant 7001–0001). V.F. and A.V.G. are grateful to Alex and Elena Kreuzberg, Viktor Lukarevsky, Alexander Tarabrin, Gochmurat Kutlyev and Sergei Morozov for their hospitality and help in field logistics.

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Manuscript received 1 June 2001, revised 7 April 2003.